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Isolation and culturing myogenic satellite cells from ovine skeletal muscle

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ABSTRACT

Sheep satellite cells more than satellite cells of the rat and mouse are similar to human satellite cells. These cells are widely used in the modeling and treatment of diseases like heart insufficiency, neurological diseases, muscular dystrophy, cerebral cell transplantation for the treatment of migraines, screening, and the production of new drugs. This study was aimed to isolate and culture primary satellite cells (PSCs) obtained from sheep fetus, and perform clonal expansion of transfected PSCs. Skeletal muscle tissues of hind limbs were collected from sheep fetuses obtained from a local abattoir. After enzymatic digestion, flasks were replaced after 3 hours to isolate non-myogenic cells, such as fibroblasts. After six days, the cells were differentiated to myoblasts. Using a differentiation medium containing the horse serum, myotube cells were observed in the flask, indicating that the cultured cells were satellite cells. The mRNA expression of the PAX7 gene was used to confirm the presence of satellite cells. In addition, the results showed that satellite cells grow in a culture medium containing 5% FBS without differentiation, while 10% FBS initiates their differentiation.

Keywords

Myoblasts, PAX7, Satellite cells, Sheep

Abbreviations

PSCs: Primary satellite cells

MDSCs: Muscle-derived satellite cells

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Introduction

Skeletal muscle tissue is composed of different cell types. The growth and development of this tissue are controlled by several mechanisms [1]. Homeostatic and regenerative replacement of skeletal muscle fibers requires the activity of a dedicated pool of self-renewing muscle-derived satellite cells (MDSCs) [2, 3]. MDSCs are small mononucleotide fusiform cells, lying between the basal lamina and the sarcolemma of muscle fibers, making them difficult to isolate [4, 5]. The intermediate filament protein, desmin, and the striated muscle actin protein, a sarcomeric actinin that is abundantly expressed in skeletal muscle cells, are involved in the movement and can be used to identify skeletal muscle cells in other tissues. Furthermore, the myogenic regulatory factors, Myf5 and MyoD1, are markers of the proliferation and differentiation of satellite cells. PAX7 also has a vital role in the maintenance of satellite cells. In zebrafish, PAX7 expression marks muscle progenitor cells, and when muscle tissue is injured, PAX7+ cells migrate around the site of injury and enter the cell cycle, while adjacent fibers up-regulate the expression of myogenic regulatory factor [6]. Immunodetection assays have shown the presence of 93% PAX7 cells and 8% MyoD cells. PAX7 marks both quiescent and activated satellite cells, whereas MyoD marks activated satellite cells only[6, 7].

In addition to MDSC's role in the acute repair of damaged muscle tissue, satellite cells are of interest in some research fields such as aging, stem cell therapy, exercise, and neuromuscular diseases [8]. The first MDSCs were found in an electron microscopic study of the peripheral region of the frog skeletal muscle fiber [9], but later the viable satellite cells were isolated from adult rat skeletal muscles [10]. Satellite cells appear in the limbs at day 17 in the ovine embryo, after primary muscle fibers have formed [11]. A subpopulation of satellite cells may be derived from a more primitive stem cell [12]. Their origin is not known, but there is evidence that they are derived from the dorsal aorta [11].

Quiescent satellite cells are characterized by the expression of PAX7 and Myf5, but not by MyoD or Myogenin. Damage to the environment surrounding satellite cells results in the deterioration of the basal lamina and their exit from the quiescent state (satellite cell activation) [13]. During regeneration, activated satellite cells could return to quiescence to maintain the satellite cell pool. This ability is critical for long-term muscle integrity [13].

Skeletal muscle satellite cells have received a great deal of attention because they directly participate in skeletal muscle differentiation and repair of adult muscle tissue. Most MDSC studies have involved mice, rats, and humans, while few MDSC studies have focused on livestock, such as cows and sheep [14]. This study aimed to establish a protocol for isolating and culturing the primary satellite cells (PSCs) obtained from ovine fetal muscle tissue.

Results

At first, different types of cells with different morphologies were observed (Figure 1-A). By placing the cells inside the flask and replacing the medium after 1h, some of the fibroblast cells attached to the flask. After 10–14 h, other cell types began to attach to the plate; some of the adherent cells had round, spindle, or polygonal shapes (Figure 1-B). Most non-specific MDSCs remained in the initial flask by transferring the medium into other flasks. After the second round of selection, the remaining cells had a completely compressed appearance and were similar to cubic cells of the transplanted tissue. Other cells, such as the nerve and the fat, were removed after the third transfer (Figure 1-C). Cells obtained from the forth and the fifth transfer were muscle cell precursors. Although their size was small, but they required 4-5 days to grow larger (Figure 1-D and E). Five days after inoculation, half of the cells were fused, had long tubular shapes and significantly larger size than single MDSCs, and many nuclei could be seen in the swollen regions of the cytoplasm. These features indicate the formation of myotubes. In the first 48 hours fibroblast cells appeared, after 96 hours myoblasts, and on the sixth day, satellite cells were observed regularly with spherical shape (Figure 1-F).

RT-PCR reactions were performed with PAX7 (satellite cell-specific markers) primers. RT–PCR results showed that PAX7 is expressed, confirming that the isolated cells were MDSCs (Figure 2).

Bacterial contamination can easily be detected after a few days. No bacterial contamination was detected by PCR analysis (Figure 3).

Figure 4 depicts the cellular-growth curves for satellite cells determined by the trypan blue assay. In this figure, the “lag-phase” was present in all cellular lines during the first 24h of incubation. After this period, the “log-phase” started to indicate that the growth was more significant for 10%FBS than the rest of cellular lines. These cell showed the highest growth rate in this experiment; whereas the growth rates of the rest of other lines were as follows: 5% FBS > 0% FBS. The 5% and 10% contained FBS manifested three phases of cell growth, while the rest of cells only manifested the “lag and death-phases”. In other words, cells in 0% FBS line never reached the “log-phase”, as it remains practically on the “lag-phase”, because the medium did not have any FBS. In the 10% FBS, cells showed the highest max value at day 4; whereas, the 5%FBS cells has the lowest max value. It can be concluded that by increasing the FBS, the growth and differentiation of the satellite cells increased. Also, we might control the differentiation of satellite cells with media containing 5% FBS, consequently cell proliferation will speed up and we may have a chance to obtain more cells from limited primary cells.

Depending on the size indicated on the lam, the diameter of the satellite cells was measured. Each degree is equal to 0.01 mm. According to Figure 5, the diameter of the satellite cell is 1.5 degrees, which is equal to 0.015 mm.

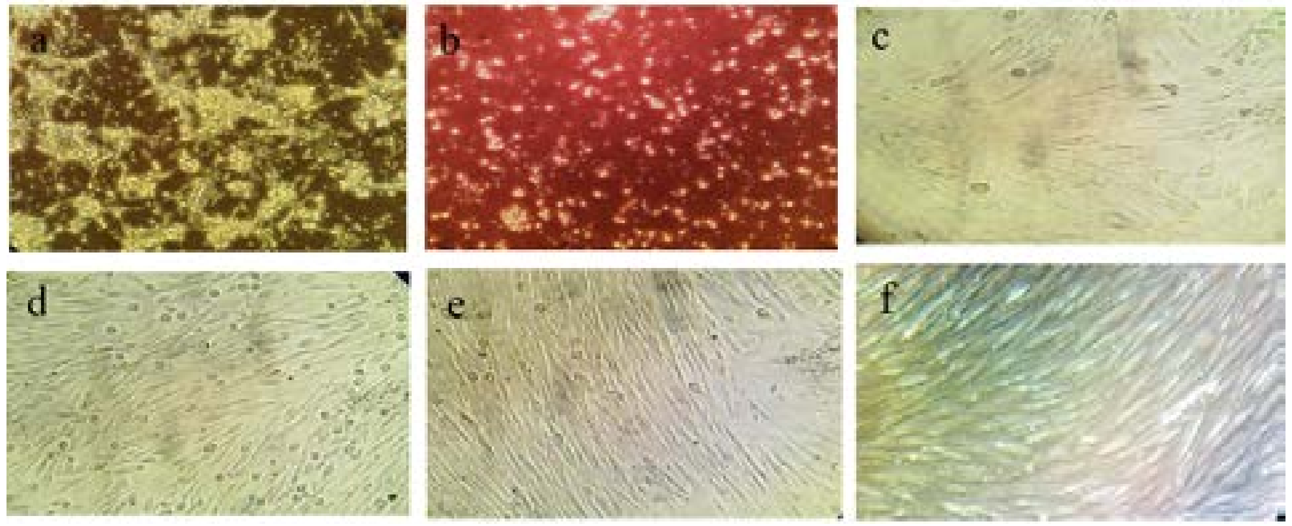

Figure1
Primary cells derived from hind limbs of sheep fetus from day one to six. a) The day-1 cultured cells including a combination of several different cell types. b) The day-1 non-specific muscle-derived satellite cells. c) The day-2 screening showing a variety of cell such as nerve and fat cells. d-e) Days 4-5, including primary skeletal muscle cells. f) The day-6 differentiated sheep skeletal muscle satellite cells.

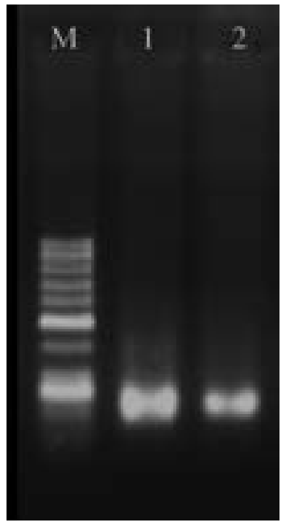

Figure 2
PAX7 was amplified with primers designed to amplify a 300-bp product (lanes 1 and 2) in the primary satellite cells. DNA size marker (M) is 1 Kbp DNA ladder.

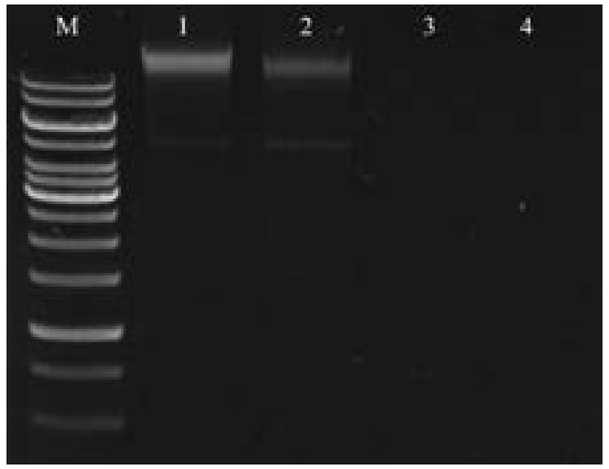

Figure 3
Agarose gel electrophoresis of PCR products for microorganism detection. Lanes 1 and 2: The genomic DNA was extracted from suspended cells in the medium, lane M: 1Kbp DNA size marker, and lanes 3 and 4 negative results of PCR reactions for Mycoplasma and bacterial contamination detection.

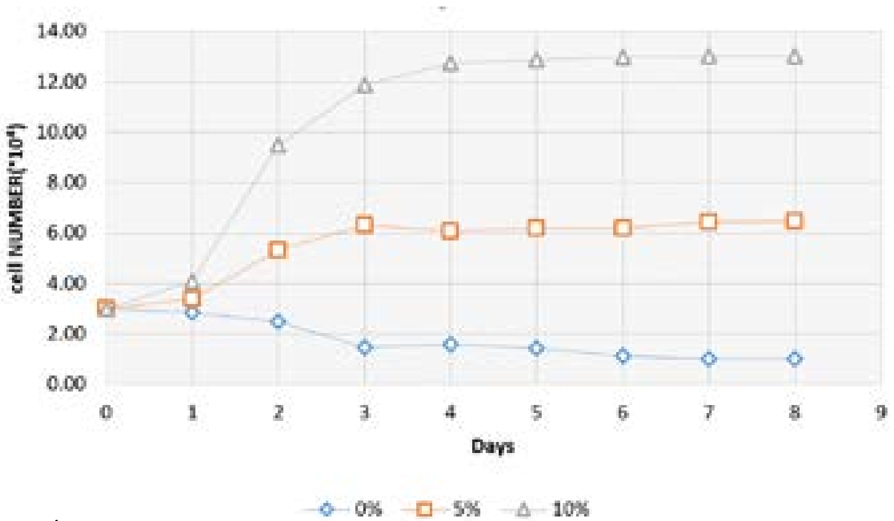

Figure 4
Cell growth curve of ovine MDSCs. The growth rate of the cells cultured in the medium containing 10% FBS was significantly higher than the cells cultured in medium containing 5% FBS.

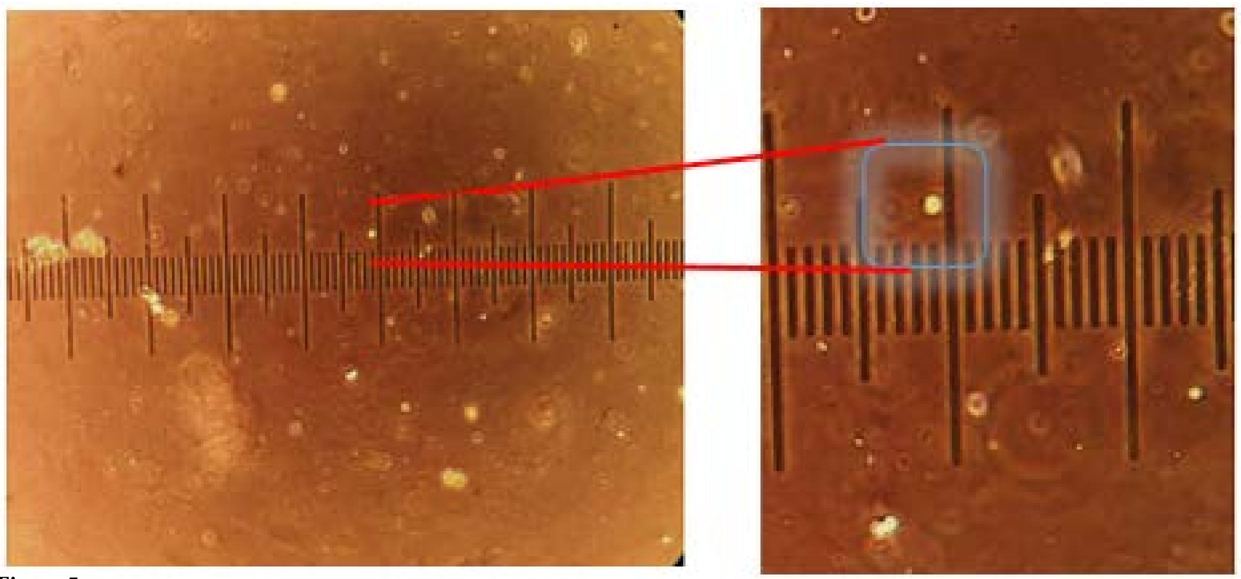

Figure 5
Morphological characteristics of isolated skeletal muscle satellite cells under the microscope. Each degree is equal to 0.01 mm.

Discussion

The purpose of this study was to establish a method for the in vitro isolation and purification of ovine MDSCs and find a more comprehensive identification method for these cells. The number of MDSCs decrease with age. We have successfully isolated sheep MDSCs, optimized their identification, and their cell proliferation and pluripotent differentiation capability assays.

Methods are being continuously updated with the developments in biotechnology [15]. The choice of methods depends mainly on the isolation scale and the subsequent experiments [17,18]. In the isolation process, 0.2% type I and IV collagenase and trypsin have been used to digest the skeletal muscle tissues. The cells have been grown in DMEM with 20% FBS and 1% penicillin/streptomycin [19, 14].

Skeletal muscle satellite cells are adult stem cells. Thus, the postnatal period is a suitable time for the isolation of skeletal muscle satellite cells. Mesires and Doumit (2002) indicated that the absolute number of these cells increased between 1 and 32 weeks of age [12]. However, the relative proportion of porcine skeletal muscle satellite cells gradually decreased from 1 to 64 weeks after birth. Satellite cells account for 30–35% of the sublaminal nuclei on myofibers in the early postnatal murine muscles, and this number declines to 2–7% in adult muscles [20]. Therefore, it is better to select newborn animals at no more than two weeks of age to obtain a high proportion of muscle satellite cells. In this study, 50-60 old-day sheep were used to isolate the skeletal muscle satellite cells and functional positive skeletal muscle satellite cells were obtained.

By adding DMEM containing 10% FBS and 2% HS, 90% of the cells in the flask differentiated to mononuclear myocytes or myotubes after four days. In adult skeletal muscle, all or most of satellite cells express the Pax3, Myf5, Barx2, M-cadherin, c-Met, α7-integrin, CD34, syndecan-3, syndecan-4, caveolin-1, Receptor Calcitonin, and Pax7. Some of the satellite cell markers (e.g. α7-integrin and CD34) are also expressed on other cell types within skeletal muscle, and thus should not be utilized alone to identify satellite cells. Pax7 is specifically expressed in satellite cells within skeletal muscle, in both quiescent and proliferating stages [13]. PAX7 is the best method to identify satellite cells. PAX7 can activate transcription in quiescent satellite cells and does not prevent the fusion of satellite-cell-derived myoblasts [21].

We successfully isolated and cultured sheep primary satellite cells via mechanical and enzymatic disaggregation. Two major approaches have been applied to isolate skeletal muscle satellite cells. The first approach was to break down the connective tissue network and myofibers to release the muscle satellite cells based on the mincing, enzymatic digestion, and repetitive trituration of the muscle mass. This was the classical and efficient method to obtain enough muscle satellite cells, although this method might obtain a heterogeneous population of precursor cells. The second approach was to isolate the muscle satellite cells from a single intact muscle fiber, which could result in relatively pure muscle satellite cells. This method has been successfully used in studies of muscle satellite cells in rats [22], mice [23], and humans [10]. Our findings provided an experimental basis for the research on ovine muscle-derived satellite cells and the methodology can be applied in other related research areas.

Materials & Methods

Reagents

The media and reagents used in this research included Dulbecco's Modified Eagle Medium, high glucose (DMEM-HG) (Gibco, Life Science, USA), fetal bovine serum (FBS) (Gibco, Life Science, USA), horse serum (HS) (Invitrogen, New Zealand), phosphate-buffered saline (PBS) (Sigma, USA), 0.25% Trypsin-ethylenediaminetetraacetic acid (Life Technologies, NY, Grand Island, USA), collagenase type I and IV (Sigma, St. Louis, MO, USA), 100x penicillin-streptomycin (10,000 U/mL) (Invitrogen, Carlsbad, CA, USA), 100% ethanol (Taghtir Khorasan, Iran), dimethyl sulfoxide (DMOS) (Sigma, USA), amphotericin (Cipla, India), and gelatin (Sigma-Aldrich, Louis, USA).

Sheep muscle tissues collection

Due to the critical stage of the skeletal muscle development at mid-gestation in sheep, muscle tissues were collected before mid-gestation from hind limbs of 50 to 60-day-old sheep fetuses [24]. Samples were kept in PBS supplemented with 10% penicillin-streptomycin and 10% amphotericin on ice before transfer to the cell culture laboratory.

Isolation and culture procedures of muscle satellite cells

The surface of the hind limbs was rinsed 3-4 times with PBS supplemented with 10% penicillin-streptomycin and 10% amphotericin. Then, the whole semitendinosus (ST) and semimembranosus (SM) muscles on the right and left legs were dissected. Visible adipose and connective tissues on the muscle mass were removed. The minced pieces of muscle were added to 6-well plates containing PBS, amphotericin, and penicillin-streptomycin. The muscle pieces were soaked in each well for 5 min to remove any surface contamination [19].

Each small tissue sample (~ $1mm^3$ cubes) was transferred into a 50 ml tube containing 5 ml of collagenase solution (DMEM containing 10% FBS and 0.2% type I and IV collagenases), and incubated in a shaking incubator at 37 °C for 90 min. Then, trypsin was added (2 times the tissue volume) and the tube was incubated for 3 min in a 37 °C incubator [25]. To neutralize the effects of trypsin, the medium containing FBS was added and the tubes were centrifugated at 500g at 4 °C for 5 min. The supernatant containing the dissociated cells was transferred onto a cell strainer (40 µm) to collect cells. The collected cells were centrifugated at 500g at 4 °C for 5 min and were separated from the supernatant. The cells were resuspended with 5 ml of 2% FBS in DMEM and transferred into

T25 cell culture flasks coated with 0.2% gelatin [16], and incubated at 37 °C under 5% CO_2 for 1 h [19, 25].

Separation of non-myogenic cells

After 1 h, the fibroblasts quickly adhere to the bottom of the cell culture flask, while the skeletal muscle satellite cells remain in the supernatant. Thus, the supernatant containing the skeletal muscle satellite cells were collected from the supernatant in a 15 mL centrifuge tube after centrifugation for 5 min at 500g. The cell pellet was washed with 10 mL of PBS, was resuspended with 5 mL 37 °C preheated DMEM containing 10% FBS, and plated in T25 cell culture flask and incubated at 37 °C under 5% CO_2 [25]. The culture medium was replaced with fresh medium every 2 days. After 6 days, the cells were differentiated [14]. Sheep MDSCs were identified by a reverse transcript PCR (RT-PCR) reaction that amplified PAX7 transcript using forward, (5'-ATTGAGGACTACAAGAGGGAAAACC-3') and reverse (5'-CTGCTTACGCTTCAGAGGGAG-3') primers.

Total RNA was extracted by the total RNA extraction kit (Parstous, Iran). One µg of total RNA was used to make cDNA by the Easy cDNA synthesis kit (Parstous, Iran). The PCR reaction was conducted in a 25 µl reaction containing: 12.5 µl 2x mastermix (Parstous, Iran), 1 µl cDNA, 1 µl primer mix (10 µM) and 10.5 µl ddH_2O. PCR program consisted of 10 min at 94 °C as initial denaturation followed by 35 cycles of 94 °C (30 sec), 57 °C (15 sec) and 72 °C (30 sec), and the final extension at 72 °C for 5 min.

MDSCs growth curves

Once the cells reached 80-90% of confluence, the cells were counted (THOMA, Germany). Then the cells were seeded at a density of 30,000 cells per well in a corning 24-well plate. Different concentrations of FBS (0, 5 and 10%) were used to grow the cells before counting on day 8 [14].

Bacterial and Mycoplasma testing

Mycoplasma contamination of cells often goes unperceived. Unlike bacteria, mycoplasmas do not cause consistently observable alterations in cell culture, such as rapid pH change or culture turbidity [26]. Therefore, the cultured cells were assessed by a DNA PCR test for the presence of both mycoplasma and bacterial species. The sequence of primers for the detection of multiple mycoplasma (including: Mycoplasma Bovis, Mycoplasma pneumonia, Candidatus Mycoplasma aurantifolia, Candidatus Phytoplasma pruni, and etc.) and bacterial species is presented in Table 1.

Morphology of satellite cells

The neobar lam was used to measure cell diameter. Cells were separated from the T25 cell culture flask by using a trypsin-EDTA solution. They were incubated for 2 min, then neutralized with a DMEM containing FBS. 100 µl of it was cast on the lam. Finally, the diameter of the cells was measured under a microscope.

Authors' Contributions

ZR and AJ contributed to the sampling and in vitro experiments, as well as preparing the first draft of the manuscript. ND helped with the cell culture and editing the final proof. HD assisted with editing the manuscript and troubleshootings of cell culture., AJ designed the experiment, edited, and finalized the manuscript.

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Competing Interests

The authors declare that they have no conflict of interest.

Table 1.
Primer sequences of multiple mycoplasma and bacterial species detection

	Primer sequence
Mycoplasma	Forward 1: 5'CGC CTG AGT AGT ACG TTC GC3'
	Forward 2: 5'CGC CTG AGT AGT ACG TAC GC3'
	Forward 3: 5'TGC CTG GGT AGT ACA TTC GC3'
	Forward 4: 5'CGC CTG GGT AGT ACA TTC GC3'
	Forward 5: 5'TGC CTG AGT AGT ACA TTC GC3'
	Forward 6: 5'CGC CTG AGT AGT ATG CTC GC3'
	Forward 7: 5'CAC CTG AGT AGT ATG CTC GC3'
	Reverse 1: 5' GCG GTG TGT ACA AGA CCC GA3'
	Reverse 2: 5'GCG GTG TGT ACA AAA CCC GA3'
	Reverse 3: 5'GCG GTG TGT ACA AAC CCC GA3'
Bacteria	Forward: 5'ACG TCR TCC MCA CCT TCC TC 3'
	Reverse: 5'GTG STG CAY GGY TGT CGT CA3'

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